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Molecular phenotyping of HCS-2/8 cells as an *in vitro* model of human chondrocytes[1]

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Summary

Objective: Cultures of primary articular chondrocytes for studying chondrocyte biology are notoriously difficult to handle. One alternative is the use of chondrocytic cell lines. Because the HCS-2/8 cells are the most widely used cell line in cartilage research, we investigated the molecular phenotype of these cells by mRNA-expression profiling.

Design: Monolayers of HCS-2/8 cells were cultured to sub-confluence, confluence and over-confluence; primary human chondrocytes were grown in monolayer culture and alginate-bead cultures and several other chondrocytic cell lines were cultured as monolayers. RNA was isolated and analyzed by cDNA array profiling using Affymetrix GeneChips (U95A/U95Av2) and quantitative PCR.

Results: Important similarities, but also remarkable differences between the HCS-2/8 cells and adult human articular chondrocytes were detected: Aggrecan and several cartilage typical collagens as well as SOX9 transcripts were strongly expressed in HCS-2/8 cells, whereas HCS-2/8 cells expressed hardly any chondrocyte-typical cartilage matrix degrading enzymes. Of all culturing conditions, clustering analysis showed that HCS-2/8 cultured at confluence are most closely related to primary chondrocytes.

Conclusion: Our study confirms how careful one needs to be in choosing *in vitro* model systems for investigating effects of interest. The major issue of chondrocyte cell lines appears to be that they mainly proliferate and show less expression of genes of matrix synthesis and turnover. A successful approach will have to select suitable chondrocyte cell lines and to validate findings obtained using primary chondrocytes. This allows to establish a reproducible *in vitro* model showing the property of interest and subsequently to relate back the obtained results to the physiologic situation.

Key words: Chondrocyte, Cartilage, Cell line, Matrix, Chondrosarcoma.

Introduction

Cultures of primary articular chondrocytes as *in vitro* model systems for studying the cellular behavior of chondrocytes as well as mechanisms of degenerative joint disease are difficult, because primary adult chondrocytes are in normal articular cartilage largely post-mitotic[1]. Thus, also after isolation of the cells from their natural protecting surroundings, chondrocytes in their native phenotype show hardly any proliferation. If they start to do so, this is usually accompanied by the loss of differentiation which involves significant changes in their molecular phenotype: classically, they stop to express cartilage collagen type II and aggrecan and initiate strongly the expression of collagen types I and III[2–4]. Together with the fact that human primary chondrocytes are difficult to obtain for all sorts of reasons including the difficulty to isolate from a single joint a significant number of cells, there exists a high need for well available cellular systems in order to simulate and investigate cellular reaction patterns in defined conditions *in vitro*. Another major problem in working with primary human adult articular chondrocytes is the high variability of reaction pattern in between different preparations obtained due to a high inter-individual variability in between different donors.

The only way to circumvent these problems appears to be the establishment of immortalized chondrocytic cell lines, which allow us to perform investigations in a largely unlimited and standardized manner. In this respect, Takigawa and colleagues established one of the first chondrocytic cell lines from a human well-differentiated chondrosarcoma[5]. The HCS-2/8 cells were described in several studies as retaining a chondrocytic morphology in long-term culture. These cells have since then been used in many studies in order to investigate cellular reaction pattern of chondrocytic cells with regard to loading[6], analysis of intracellular signal transduction pathways[7,8] and in particular effects of connective tissue growth factor (CTGF)[9–12] and TNFα[13]. Recently, a rheumatoid-arthritis specific antigen was reported to be expressed by HCS-2/8 cells[14].

Because the HCS-2/8 cells are common tools in cartilage research as described above, we investigated in this study

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the molecular phenotype of these cells in different culture conditions (growing densities) by comprehensive mRNA-expression profiling. We used Affymetrix high-density oligonucleotide microarrays to accomplish the measurement of ~12,600 genes in parallel and to generate an in-depth expression profile of HCS-2/8 cells and compared these to the profiles of other human chondrocyte cell lines and of primary human adult articular chondrocytes. In addition, we used online quantitative PCR to confirm part of these findings.

Material and methods

CULTURE OF HCS-2/8 CELLS

The human HCS-2/8 chondrosarcoma cell line (around passage 50–55) was cultured in DMEM medium (PAA, Austria) supplemented with 20% fetal bovine serum (Gibco BRL, Germany) and with (real-time PCR) and without (array analysis) 50 μg/ml ascorbate (Sigma, Germany) in humidified atmosphere 5% CO_2 in air at 37°C as described[5]. Cells were seeded with densities of $1 \times 10^5/cm^2$ and grown for 3 days to obtain sub-confluent stage cultures, $2 \times 10^5/cm^2$ and cultured for 7 days to obtain confluent stage cultures, and $6 \times 10^5/cm^2$ and grown for 10 days for over-confluent stage cultures. AG, SG and SW1353 chondrosarcoma cell lines were cultured as described[15] and harvested at confluence. Immortalized C28I2 and C28a4 chondrocyte lines were cultured as described by Goldring *et al.*[16] and harvested just at the point when cells reached confluence.

ISOLATION AND CULTURE OF PRIMARY HUMAN CHONDROCYTES

Normal human knee articular cartilage were obtained from two donors at autopsy (59 years male; 71 years male), within 48 h of death. Cartilage pieces were finely chopped and chondrocytes were enzymatically isolated as described previously[17]. Chondrocytes were cultured in alginate beads as described previously[18]. Cultures were maintained for 48 h in serum-free DMEM/F12 medium (Gibco BRL, Germany) supplemented with 1% penicillin/streptomycin solution (Gibco BRL, Germany) and 50 μg/ml ascorbate (Sigma, Germany) and 10% fetal calf serum (Biochrom, Germany). After 2 days the cells were washed in sterile PBS, lysed in 350 μl lysis buffer/10^6 cells (Qiagen GmbH, Germany) and stored at −80°C.

RNA ISOLATION

At the end of the culture period, cells were washed in DEPC-PBS and lysed in Qiagen RNA lysis buffer (350 μl/10^6 cells). RNA was isolated according to the manufacturer's protocol (Qiagen Mini RNeasy total RNA isolation kit; Qiagen GmbH, Germany). The quality of the isolated RNA was assessed by ethidium bromide staining on agarose gels and the concentration was determined by comparison to an RNA standard of the known concentration (Peqlab RNA marker, Peqlab Erlangen, Germany). Additionally, spectral measurements (260/280 nm) were performed.

REVERSE TRANSCRIPTION

First strand cDNA was synthesized using 1 μg of total RNA, 400 U M-MLV Reverse Transcriptase, RNase H Minus (Promega, Germany), 2 mM dNTPs (Roth) and 200 ng random primers (Promega, Germany) in a total volume of 40 μl.

TAQMAN-PCR

TAQMAN-PCR was used to detect human COL2, COL10, aggrecan, MMP-1, -3, -13 as well as ADAMTS-1, -4 and -5, beta-actin and GAPDH. The primers (MWG Biotech, Germany) and TAQMAN probes (Eurogentec, Belgium) were designed using PRIMER EXPRESS™ software (Perkin Elmer)[17,19]. In order to be able to obtain quantifiable results for all genes specific standard curves using sequence specific control probes were performed in parallel to the analyses. Thus, for each gene a gene-specific cDNA fragment was amplified by the gene-specific primers (Table I) and cloned into pGEM T Easy (Promega, Mannheim, Germany) or pCRII TOPO (Invitrogen, Karlsruhe, Germany). The cloned amplification product was sequenced for confirmation of correct cloning. Cloned standard probes were amplified using the QIAfilter Midi Plasmid Kit (Qiagen, Germany) and linearized by restriction digest. Linearized standard probes were gel purified using the QIAquick Gel Extraction Kit (Qiagen). Purified probes (fragments) were quantified using a fluorometric assay (Picogreen, Molecular Probes, Oregon, USA). Concentrations were confirmed (checked) by measuring the absorbance at 260 nm in an UV-spectrophotometer and compared with DNA bands of known concentration (MassRuler™ DNA Ladder, MBI Fermentas, Germany) in an ethidium bromide stained agarose gel.

For the standard curves concentrations of 10, 100, 1000, 10,000, 100,000, as well as 1,000,000 molecules per assay were used (all in triplicates).

A separate master-mix was made up for each of the primer pairs and contained a final concentration of 200 μM NTPs, 600 nM Roxbuffer and 100 nM TAQMAN probe. For all genes the final reaction mix contained besides cDNA and 1 U polymerase (Eurogentec, Belgium) forward and reverse primers, the corresponding probes, and $MgCl_2$ at concentrations given in Table I. All experiments were performed in triplicates.

GENECHIP EXPERIMENTS

8–10 μg of total RNA were reverse transcribed with T7-$(dT)_{24}$ primers (Qiagen) and 400 U SuperScript II RT (Invitrogen Lifetechnologies, Germany) at 42°C. Second strand synthesis was done with *Escherichia coli* DNA polymerase I, *E. coli* DNA ligase and *E. coli* RNAse H at 16°C, cDNA fragments were blunt-ended with T4 DNA polymerase. The cDNA was purified by phenol–chloroform extraction, precipitated with ethanol and resuspended in 12 μl RNAse-free water. Six microliters of the cDNA was subjected to an *in vitro* transcription reaction using a Bioarray high yield RNA transcript labeling kit (Enzo Diagnostics). The cRNA was purified using RNeasy spin columns (Qiagen) and fragmented by metal induced hydrolysis for 35 min at 94°C. Fifteen micrograms of fragmented cRNA was hybridized together with control transcripts to the U95A or U95Av2 GeneChips (Affymetrix, USA). Washing and staining of the GeneChips were done according to the instructions of the manufacturer using the Affymetrix Fluidics station and chips were scanned with a confocal scanner (Hewlett–Packard Gene array scanner). All procedures are described in detail in the Affymetrix Technical Manual.

Table I
Sequences and concentrations of primers and probes as well as magnesium conditions for quantitative online-PCR experiments

	Accession No.	Primer	nM	Probe	Mg mM
GAPDH	NM_002046	fw: GAAGGTGAAGGTCGGAGTC	50	CAAGCTTCCCGTTCTCAGCC	5.5
		rv: GAAGATGGTGATGGGATTTC	900		
Aggrecan	NM_013227	fw:ACTTCCGCTGGTCAGATGGA	50	CCATGCAATTTGAGAACTGGCGCC	6
		rv: TCTCGTGCCAGATCATCACC	50		
COL2A1	NM_001844	fw:CAACACTGCCAACGTCCAGAT	50	ACCTTCCTACGCCTGCTGTCCACG	5.5
		rv: CTGCTTCGTCCAGATAGGCAAT	300		
COL1A1	NM_000088	fw: AGGGCCAAGACGAAGACATC	50	AATCACCTGCGTACAGAACGGCCTCA	6.5
		rv: AGATCACGTCATCGCACAACA	300		
MMP-1	NM_002421	fw: CTGTTCAGGGACAGAATGTGCT	300	ACGGATACCCCAAGGACATCTACAGCTCC	6.5
		rv: TCGATATGCTTCACAGTTCTAGGG	900		
MMP-3	NM_002422	fw: TTTTGGCCATCTCTTCCTTCA	900	AACTTCATATGCGGCATCCACGCC	4
		rv: TGTGGATGCCTCTTGGGTATC	50		
MMP-13	NM_002427	fw: TCCTCTTCTTGAGCTGGACTCATT	900	TCCTCAGACAAATCATCTTCATCACCACCAC	7
		rv: CGCTCTGCAAACTGGAGGTC	50		
ADAMTS-1	NM_006988	fw: GCCAAAGGCATTGGCTACTTC	900	CGTTTTGCAGCCCAAGGTTGTAGATGGT	7
		rv: GTGGAATCTGGGCTACATGGA	900		
ADAMTS-4	AF148213	fw: TGCCCGCTTCATCACTGA	900	ACAGTGCCCATAGCCATTGTCCAGGA	6
		rv: CAATGGAGCCTCTGGTTTGTC	50		
ADAMTS-5	AF142099	fw: CGCTGCCACCACACTCAA	300	AAGTGGCAGCACCAACACAACCAGC	4.5
		rv: CGTAGTGCTCCTCATGGTCATCT	900		
Ki-67	NM_002417	fw: CAGTGATCAACGCCGTAGGTC	900	CTTCCAGCAGCAAATCTCAGACAGAGGTTC	6
		rv: TCGGCTGATAGACACTCTCTTTTG	900		
Sox9	Z46629	fw: ACACACAGCTCACTCGACCTTG	900	TTAGGATCATCTCGGCCATCGTCGC	7
		rv: GGAATTCTGGTTGGTCCTCTCTT	50		

ANALYSIS OF GENECHIP MICROARRAY DATA

The performance of the chip experiments was controlled by the Affymetrix Microarray Suite (Version 4.0.1) software. Raw data were then imported into the Rosetta Resolver® software (version 3.1, Rosetta Biosoftware) and analyzed further using the provided functionality. Replicate experiments were first correlated to each other and then combined to obtain more robust statistical values (detection *P*-values). The detection *P*-values are an estimation to assess whether a gene is detected as expressed compared to background noise. The algorithm therefore compares the intensity distribution of the signal with the intensity distribution of negative control signals[20]. As a result to each gene a significance value is assigned, which indicates the probability that a detected gene is expressed.

Results

GENECHIP AND QPCR BASED CHARACTERIZATION OF HCS-2/8—INFLUENCE OF CULTURE DENSITY—GENERAL GENE EXPRESSION ANALYSIS

For gene expression profiling of HCS-2/8 and primary chondrocytes we used the Affymetrix GeneChip U95Av2 containing 12,625 probe sets. The whole list of expression values from the profiling experiments are available online as supplementary material. The 20 most abundant transcripts from each HCS-2/8 culture conditions are listed in Table II, many of them belonging to the basic cellular machinery of the cells (i.e., ribosomal proteins etc.).

In vitro culture conditions such as the cell density in monolayer cultures lead to a different morphological phenotype of HCS-2/8 cells, but the corresponding molecular changes are only partially revealed[21]. We therefore performed in parallel two independent cultures of HCS-2/8 cells and gained expression profiles from cells in sub-confluent, confluent, and over-confluent stages. As shown in Table III, ~2700–5700 genes were detected in each culture condition using a threshold detection *P*-value below 0.05. The profiles derived from the same culture density showed always a very significantly higher correlation than profiles obtained from different culture densities [Table III and Fig. 1(a)] indicating reproducible differences in gene expression levels between these conditions. This was further supported by hierarchical cluster analysis of all six profiles. As shown in the dendrogram [Fig. 1(b)] the replicates from the same culture condition clustered together and, thus, were most similar to each other.

Next, we identified differentially expressed genes based on an at least twofold difference in intensity and a corresponding *P*-value of less than 0.001 as a threshold. Under these conditions 608 genes and 1577 genes were significantly different when confluent and over-confluent HCS-2/8 cells were compared to the sub-confluent ones, respectively. Among these genes, especially among the most strongly repressed genes in confluent and over-confluent stages, a high percentage were cell cycle control genes, an excerpt of these is listed in Table IV and discussed below. This was expected since the cells proliferate at a much decreased rate when they reach confluence[21].

GENE EXPRESSION PROFILING OF HCS-2/8 CELLS—COMPARISON TO ISOLATED NORMAL ADULT ARTICULAR CHONDROCYTES—ANALYSIS OF MARKER GENES OF CHONDROCYTIC DIFFERENTIATION

As a next step, we were interested in comparing the levels of mRNA expression of HCS-2/8 cells with isolated primary human adult articular chondrocytes. This revealed not only important similarities in mRNA levels in between the HCS-2/8 cells and freshly isolated adult human articular chondrocytes, but also remarkable differences (Table V). Steady state levels of aggrecan transcripts were considerable in sub-confluent cells, increased in confluent HCS-2/8 and remained high also in the over-confluent stage.

Table II

Table showing the 20 most strongly detected genes in HCS-2/8 cells in the culture conditions tested: sub-confluent, confluent and over-confluent (a–c) and in primary human chondrocytes (PHC) (d) cultured in alginate. The genes were ranked by the mean of normalized expression levels

a			b			c			d		
Sub-confluent	Accession No.	Intensity	Confluent	Accession No.	Intensity	Over-confluent	Accession No.	Intensity	PHC	Accession No.	Intensity
Ribosomal protein L28	U14969	21,038	Clusterin	M25915	29,463	Annexin A2	D00017	26,770	Clusterin	M25915	55,489
Clusterin	M25915	20,484	Annexin A2 pseudogene 3	M62895	28,512	Clusterin	M25915	24,982	Ferritin light polypeptide like 1 (FTL)	AL031670	46,338
Annexin A2 pseudogene 3	M62895	20,337	GAPDH	U34995	27,855	Coupling protein G(s) alpha-subunit (alpha-S1)	X04409	20,038	Ferritin H	J04755	43,156
Laminin receptor 1	M14199	18,877	Ferritin H	J04755	22,534	Ribosomal protein L28	U14969	19,682	Biglycan	J04755	43,156
Ribosomal protein S5	U14970	18,505	Ribosomal protein L28	U14969	22,381	Ribosomal protein L8	Z28407	19,185	TIMP1	D11139	40,858
GAPDH	U34995	17,939	Ribosomal protein L8	Z28407	20,833	Ubiquitin C	AB009010	19,179	Elongation factor 1 alpha	J04617	40,731
Ribosomal protein S20	L06498	17,801	Biglycan	J04599	20,806	Ribosomal protein L37a	L06499	18,866	Interferon-inducible protein 9-27	J04164	38,052
Ribosomal protein L8	Z28407	17,577	Elongation factor 1 alpha	J04617	20,436	Ferritin H	J04755	18,555	Ribosomal phosphoprotein P1	M17886	37,793
Gamma actin	X04098	17,452	Ribosomal protein L37a	L06499	20,243	Ribosomal protein S2	X17206	18,063	Chitinase 3-like 1 (YKL-40)	Y08374	37,730
Cyclophilin	X52851	17,307	Ribosomal protein L41	Z12962	20,210	GAPDH	U34995	17,976	Ribosomal protein L37a	L06499	37,407
Pyruvate kinase	M26252	16,957	Ribosomal protein S5	U14970	20,205	Ribosomal protein S20	L06498	17,958	Fibronectin	M10905	36,961
Ribosomal protein S2	X17206	16,843	Ribosomal protein S2	X17206	20,097	Ribosomal protein L41	Z12962	17,933	GAPDH	U34995	36,526
Coupling protein G(s) alpha-subunit (alpha-S1)	X04409	16,643	Ribosomal protein S20	L06498	19,764	Ribosomal protein S5	U14970	17,522	Chitinase 3-like 2 (YKL39)	U58515	34,900
Ornithine decarboxylase antizyme	D78361	16,333	Ubiquitin C	AB009010	19,104	Biglycan	J04599	17,097	Annexin A2 pseudogene 3	M62895	34,227
Elongation factor 1 alpha	J04617	16,235	Coupling protein G(s) alpha-subunit (alpha-S1)	X04409	18,696	Ornithine decarboxylase antizyme	D78361	16,859	Laminin receptor 1	M14199	32,288
Ribosomal protein S11	X06617	16,135	Prolyl 4-hydroxylase beta-subunit	M22806	18,583	Ribosomal phosphoprotein P1	M17886	16,698	Ribosomal protein L41	Z12962	31,549
Ferritin light polypeptide like 1 (FTL)	AL031670	16,102	Ribosomal phosphoprotein P1	M17886	18,512	Elongation factor 1 alpha	J04617	15,657	alpha 1-antitrypsin (SERPINA1)	X01683	30,888
Ferritin H	J04755	16,101	Laminin receptor 1	M14199	18,484	Ribosomal protein S17	M13932	15,330	Metallothionein 1E	R92331	30,666
Ribosomal phosphoprotein P1	M17886	15,989	Ribosomal protein L13a	X56932	18,466	junD	X56681	14,796	Interferon induced transmembrane protein 3	X57352	30,392
RHO A proto-oncogene	L09159	15,945	Serpine E2 (plasminogen activator inhibitor type 1)	AI743134	17,884	Ribosomal protein L13a	X56932	14,691	Ribosomal protein S2	X17206	30,010

Table III

Correlation of GeneChip profiles. Replicates for primary human articular chondrocytes (PHC) (two donors) and biological replicates of HCS-2/8 cells (duplicate cultures for each stage) for three culture conditions (_c: confluent; _s: sub-confluent; _o: over-confluent) were correlated to each other. The number of genes (N) called absent or present in the different array profiles according to the statistical analysis software are given based on a statistical detection threshold of $P < 0.05$

	PHC (T258)	PHC (T259)	HCS_s1	HCS_s2	HCS_c1	HCS_c2	HCS_o1	HCS_o2
N present	3234	2737	5667	5041	4631	4216	4600	4344
N absent	9391	9888	6958	7584	7994	8409	8025	8281
Correlation coefficient (*R*)	0.989		0.992		0.992		0.993	

Collagen type II expression was found only at a very low level with a slight increase in the confluent and over-confluent cultures. Thus, whereas levels of aggrecan expression in HCS-2/8 cells were close to primary cells, much less collagen type II was expressed in HCS-2/8 cells compared to isolated primary chondrocytes. Both results were confirmed by quantitative PCR [Table V(b)]. Other typical gene products of articular chondrocytes such as

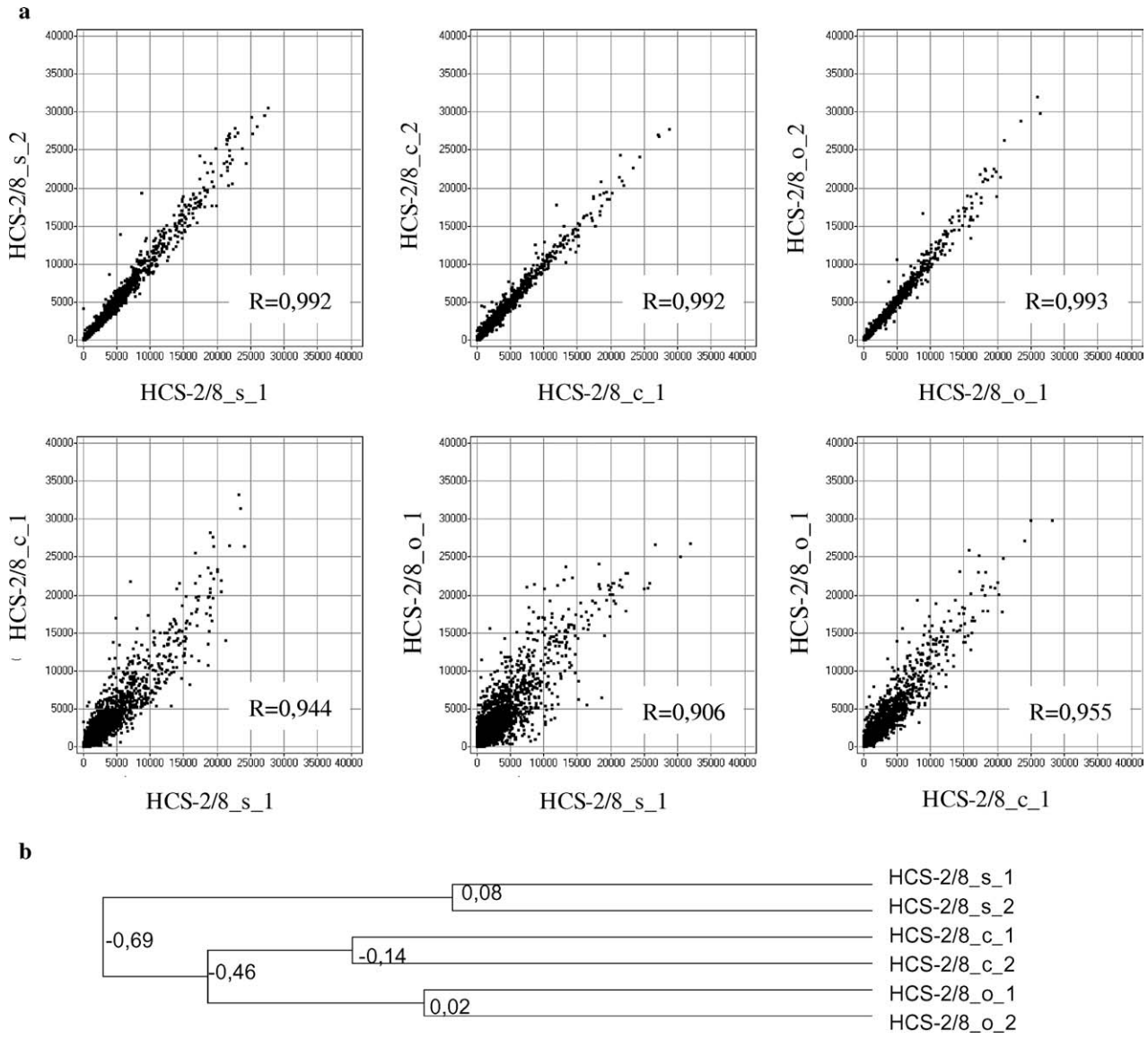

Fig. 1. Correlation of different HCS-2/8 culture conditions: (A) Intensity diagrams of profiles derived from sub-confluent (HCS-2/8_s), confluent (HCS-2/8_c), and over-confluent (HCS-2/8_o) HCS-2/8 culture conditions. Each diagram shows a comparison of two profiles; a gene is represented by a square and plotted according to it's intensity value in the first and second profiles. The intensity range is from 0 to 40,000 (rel. units) on both axes. The correlation value *R* for the gene intensities has been calculated for each comparison of two profiles. The comparisons of two profiles from the same culture conditions (interexperimental variability) show a higher correlation (upper row) than profiles derived from different HCS-2/8 culture stages (lower row). (B) Hierarchical clustering of HCS-2/8 profiles derived from the different culture conditions. An agglomerative clustering algorithm with Euclidean distance as a metric was chosen. In order to combine groups complete linkage was selected. Given are correlation values for paired groups.

Table IV
Cell cycle related genes with significant regulation in HCS-2/8 cells in dependence to the culture density. (R c/s: ratio confluent vs sub-confluent; R o/s over-confluent vs sub-confluent)

Affymetrix qualifier	Sequence description	Accession No.	Ratio c/s	*P*-value	Ratio o/s	*P*-value
1911_s_at	Growth arrest and DNA-damage-inducible protein (GADD45)	M60974	2.4	2.86E−03	9.3	<1.0E−30
37028_at	Growth arrest and DNA-damage-inducible protein (GADD34)	U83981	2.6	8.47E−04	7.9	<1.0E−30
635_s_at	Protein phosphatase 2A regulatory subunit B56-beta	L42374	1.9	0.06	6.8	2.55E−08
2031_s_at	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	U03106	2.1	<1.0E−30	4.5	<1.0E−30
40235_at	Activated p21cdc42Hs kinase (ack)	L13738	1.7	0.01	4.2	2.04E−13
36634_at	btg2	U72649	1.9	6.75E−04	3.7	<1.0E−30
1860_at	bcl2	U58334	1.6	0.39	3.4	1.00E−11
36650_at	CyclinD2	D13639	2.4	2.05E−03	3.1	2.71E−05
38326_at	G0S2	M69199	2.3	0.13	2.8	5.11E−03
37294_at	btg1	X61123	1.9	2.24E−14	2.6	3.02E−26
1924_at	Cyclin H	U11791	1.3	0.05	2.5	6.18E−21
1824_s_at	Proliferating cell nuclear antigen (PCNA)	J05614	0.35	5.32E−05	0.27	8.68E−25
1536_at	cdc6-Related protein	U77949	0.17	1.51E−06	0.14	3.42E−05
1925_at	Cyclin F	Z36714	0.13	4.78E−03	0.13	1.55E−04
35249_at	Cyclin E2	AF091433	0.14	5.38E−26	0.13	5.93E−30
1599_at	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	L25876	0.12	<1.0E−30	0.13	<1.0E−30
40041_at	Retinoblastoma-associated protein HEC	AF017790	0.13	2.60E−16	0.11	2.13E−14
1803_at	cdc2 (cell division cycle 2. G1 to S and G2 to M)	X05360	0.17	5.83E−23	0.10	<1.0E−30
1721_g_at	mad2 (mitotic arrest deficient yeast homolog-like 1)	U65410	0.15	2.17E−09	0.10	<1.0E−30
40549_at	cdk5	L04658	0.34	2.77E−07	0.10	6.87E−21
34852_g_at	Serine/threonine kinase 6	AF011468	0.19	<1.0E−30	0.08	1.02E−30
418_at	Ki-67	X65550	0.26	<1.0E−30	0.08	<1.0E−30
37458_at	cdc45-related protein	AJ223728	0.11	4.85E−23	0.08	<1.0E−30
1178_at	Dihydrofolate reductase	NM_000791	0.15	6.70E−13	0.06	<1.0E−30
32263_at	Cyclin B2	AL080146	0.05	<1.0E−30	0.05	2.67E−30
40145_at	DNA topoisomerase II	AI375913	0.12	<1.0E−30	0.04	<1.0E−30
1945_at	Cyclin B1	M25753	0.10	<1.0E−30	0.03	<1.0E−30
38414_at	cdc20 (cell division cycle 20 S. cerevisiae homolog)	U05340	0.10	<1.0E−30	0.03	<1.0E−30
910_at	Thymidine kinase	M15205	0.10	<1.0E−30	0.02	<1.0E−30

collagen types VI, IX, XI, and XVI as well as proteoglycan link protein and small proteoglycans (biglycan, fibromodulin) were also strongly expressed by HCS-2/8 cells similar to primary human articular chondrocytes. Expression of collagen type X, a marker of hypertrophic chondrocytes, was very low in any culture condition and only slightly increased with increasing culture density (Table V). Thus, overall only a very limited shift in the phenotype towards chondrocyte hypertrophy of the HCS-2/8 cells was observed in the different culture conditions. Also, long-term high-density cultures did not reveal significant alterations in collagen type X expression (own unpublished results).

Of note, also other marker genes typical for chondrocytes were expressed: thus, significant levels of CDRAP and SOX9 were detected. Steady state levels of SOX9 transcripts were significant in sub-confluent cells, increased in confluent HCS-2/8 and were high also in the over-confluent stage, similar to the SOX9 in primary human articular chondrocytes. SOX9 is known to be crucial for the chondrocytic phenotype in fetal[22] and presumably also adult chondrocytes[23].

GENE EXPRESSION PROFILING OF HCS-2/8 CELLS—COMPARISON TO ISOLATED NORMAL ADULT ARTICULAR CHONDROCYTES—CATABOLIC GENES

A severe alteration in gene expression levels in between the HCS-2/8 cells and physiological chondrocytes was found with regard to cartilage matrix degrading enzymes. MMP-1, -3, and -13 mRNAs were detectable, but under most conditions only by qPCR due to their low expression levels in HCS-2/8 in general. Thus, MMP-1, -3, and -13 mRNA levels were decreased at least by an order of magnitude compared to physiological chondrocytes [Table V(b)]. Among the cartilage matrix degrading ADAMTS proteases (these were not represented on the U95Av2 GeneChips and were due to their high importance for cartilage degradation additionally analyzed by quantitative

Table V

Table listing a comparative analysis of mRNA-expression levels of typical gene products of articular chondrocytes in HCS-2/8 (_s: sub-confluent; _c: confluent; _o: over-confluent) vs normal primary human chondrocytes ("PHC") as detected by (a) oligochip array and (b) qPCR analysis

(a) Oligochip array analysis Gene	Accession No.	HCS-2/8_s (intensity)	HCS-2/8_c (intensity)	HCS-2/8_o (intensity)	PHC (intensity)
Aggrecan	M55172	6497	10,424	9750	15,186
COL1A1	Y15915	469	406	662	567
COL2A1	L10347	45	167	123	22,562
COL3A1	X14420	3085	2937	209	3685
COL6A1	X15880	4152	5212	3861	5279
COL6A2	X15882	3534	4702	4777	10,880
COL6A3	X52022	4041	4323	2774	6963
COL9A3	L41162	7491	13,487	11,463	2449
COL10A1	X60382	187	205	200	1344
MMP-1	M13509	56	86	−15	13,122
MMP-2	M55593	245	336	211	687
MMP-3	X05232	361	64	26	27,047
MMP-13	X75308	102	38	−23	5603
MMP-14	X83535	350	320	109	305

(b) qPCR analysis Gene	Accession No.	HCS-2/8 (relative to GAPDH)	PHC (relative to GAPDH) beads	PHC (relative to GAPDH) monolayers
Aggrecan	NM_013227	3.80	0.7	0.95
Col1A1	NM_000088	0.0014	0.011	0.114
Col2A1AB	NM_001844	0.0009	1.012	3.3
Col2A1A	L10347	0.0002	0.143	0.229
Col10A1	NM_000493	0.0003	0.036	0.014
ADAMTS-1	NM_006988	0.050	0.130	0.134
ADAMTS-4	AF148213	0.0007	0.0009	0.001
ADAMTS-5	AF142099	0.002	0.295	0.069
MMP-1	NM_002421	0.018	1.20	0.37
MMP-3	NM_002422	0.017	62.9	37
MMP-13	NM_002427	0.007	0.955	0.121
SOX9	Z46629	0.11	0.18	0.09

PCR), ADAMTS-1 was most abundant with highest expression in the sub-confluent stage, ADAMTS-4 and -5 were very low (<0.005/GAPDH) in all culture conditions (Table VI). Altogether, ADAMTS-1 and -4 were expressed by the HCS-2/8 cells in the same order of magnitude than by physiological chondrocytes.

GENE EXPRESSION PROFILING OF HCS-2/8 CELLS—CELL CYCLE CONTROL GENES

Proliferating activity of HCS-2/8 cells was markedly reduced when the cells reached confluence and subsequently became over-confluent ([21], data not shown). This is reflected by the strongly increased expression of GADD45 and GADD34 (Table IV), known to be up-regulated in growth-arrested cells. Increased expression of several other genes was also detected like the regulatory subunit B56, the Ser/Thr protein phosphatase subunit 2a or the GO/G1 switch gene (G0S2), which are associated with growth and differentiation processes[24,25]. Also, the well-characterized inhibitor of a cyclin-dependent kinase p21 was found and the antiproliferative proteins BTG1 and BTG2. On the other hand, several cell cycle promoting genes were down-regulated in confluent and over-confluent stages (Table IV). Among these were several kinases (cdc2, cdk5), major cyclins of eukaryotes (cyclin B1, B2, cyclin E, cyclin F) as well as enzymes of DNA replication (dihydrofolate reductase and topoisomerase II). Also proliferation-associated genes PCNA and Ki-67 were clearly down-regulated as shown by cDNA array analysis as well as quantitative PCR (Tables IV and VI; Fig. 2).

GENE EXPRESSION PROFILING OF HCS-2/8 CELLS—COMPARISON OF THE DIFFERENT CULTURE CONDITIONS WITH RESPECT TO THE CHONDROCYTIC PHENOTYPE

Hierarchical clustering based on the expression of a large number of (significantly detected [*P*-value <0.05]) genes of the profiles from cultured primary articular human chondrocytes derived from two individuals and from the profiles

Table VI

Table presenting the expression values of a subset of anabolic and catabolic marker genes of cartilage matrix turnover as well as proliferation-associated antigen Ki-67 and chondrogenic differentiation factor SOX9 in HCS-2/8 cells in sub-confluent, confluent and over-confluent culture conditions using quantitative PCR analysis (shown are always the average values/GAPDH of three replicates of two independent cultures and measurements)

	Actin	Aggrecan	COL2	COL10	ADAMTS-1	ADAMTS-4	ADAMTS-5	MMP-1	MMP-3	MMP-13	Ki-67	SOX9
Sub-confluent	7.5	1.4	0.0002	0.0002	0.12	0.0011	<0.0001	0.007	0.034	0.008	0.016	0.05
Confluent	5.3	3.8	0.0009	0.0003	0.05	0.0007	0.002	0.018	0.017	0.007	0.004	0.11
Over-confluent	5.0	11.3	0.0008	0.0004	0.06	0.0002	0.003	0.005	0.016	0.004	0.006	0.31

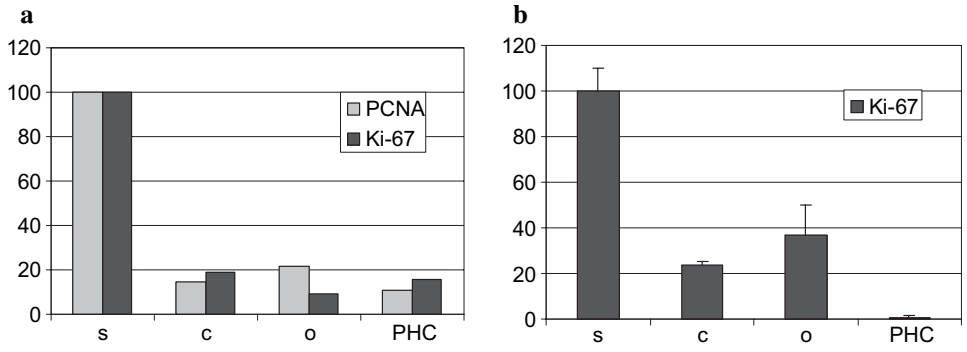

Fig. 2. Correlation of the expression of proliferation-associated antigens Ki-67 (A: cDNA array analysis, B: qPCR) and PCNA (A: cDNA array analysis) in HCS-2/8 cells in the different culture conditions (given are arbitrary values adapted to the maximal value as 1 (s: sub-confluent, c: confluent, o: over-confluent). Given are arbitrary units with sub-confluent culture levels set to 100 (%).

of the HCS-2/8 cells cultured in the different culture conditions provided a clear separation of the primary chondrocytes from the HCS-2/8 profiles, but did not resolve the relatedness of the culture stages to the primary cells (data not shown). Therefore, we made a selection of those genes, which discriminated the three HCS-2/8 stages by performing an ANOVA-test treating sub-confluent, confluent and over-confluent profiles as three separate groups. The profiles were then clustered using the 587 most significant genes (according to a cut-off value of $P < 10^{-9}$) and principal component analysis as clustering algorithm. The largest variance of the data set (principal component 1) is attributed to the different HCS stages and the second largest variance (principal component 2) is identified for PHC and the sub-confluent HCS-2/8 cells. In the coordinate system of the first two principal components (Fig. 3) the confluent culture of HCS-2/8 is most closely related to the primary chondrocytes used in this study (Fig. 3).

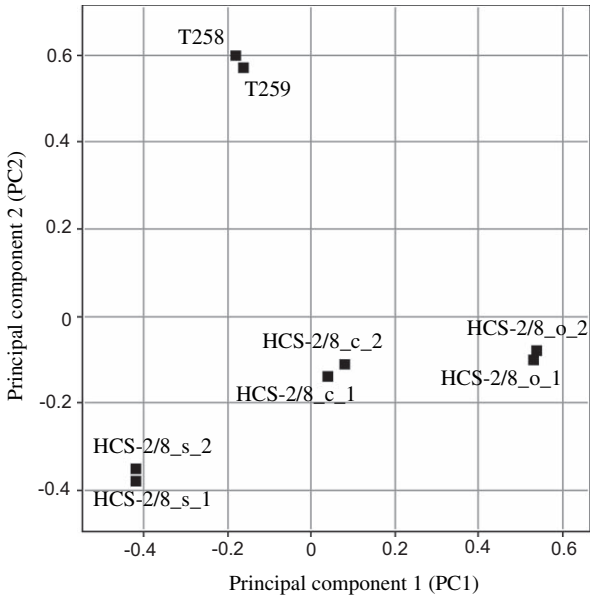

Fig. 3. Visualization of gene expression profiles from three HCS-2/8 culture stages and primary human chondrocytes after principal component analysis. Duplicate profiles of HCS-2/8 in sub-confluent [s], confluent [c] and over-confluent [o] stages and two profiles from primary human chondrocytes (samples T258, T259) were subject to a principal component analysis. The first principal component accounts for the highest variance in the data set and the succeeding principal components account for as much of the remaining variance as possible. The profiles (squares) are then shown in the space of the first two principal components (*x*- and *y*-axes, respectively) in order to infer the relatedness of the profiles. The first principal component (PC1) indicates the difference in between the various stages of the HCS-2/8 cell line. It clearly separates the most distant stages, sub-confluent and over-confluent, and places the confluent stage between the other two. Within the first principal component the primary chondrocytes are located nearly in between the sub-confluent and confluent stages of HCS-2/8, with a slight orientation towards the confluent stage. The second principal component represents the difference between the primary chondrocytes and the sub-confluent stage of HCS-2/8 cells. This component clearly indicates that the profiles of confluent and over-confluent stages of the HCS-2/8 cell line are closer to the primary chondrocytes than the sub-confluent stage. The difference is even clearer as soon as the first two principal components are taken into account. The two-dimensional plot indicates that the primary chondrocytes are more similar to the confluent stage of HCS-2/8 than to the sub- and over-confluent stages of the HCS-2/8 cells.

COMPARISON OF CHONDROCYTIC CELL LINES AND PRIMARY HUMAN CHONDROCYTES

Finally, we have cultured several other human chondrocyte cell lines as monolayers and isolated RNA for the generation of GeneChip profiles. C28I2 and C28a4 are SV40 T-antigen transformed chondrocytes[26] and AG, SG[15] and SW1353 are chondrosarcoma derived cell lines similar to the HCS-2/8 cells. Hierarchical clustering of all profiles showed that HCS-2/8 cells are most closely related to the primary chondrocytes from all cell lines studied (Fig. 4). This is clearly the case for molecules such as aggrecan, which are expressed in HCS-2/8 cells at a much higher level than in the other cell lines and in a level comparable to physiological chondrocytes. In contrast, none of the cell lines investigated expressed significant amounts of collagen type II mRNA. However, this is clearly a general view and in different aspects different cell lines might reflect best the physiological situation: thus, HCS-2/8 cells are responsive to BMP-7, but not to Il-1β (own unpublished data), whereas Goldring and colleagues were able to report an Il-1β response in the C28/I2 and T/C28a4 cell lines[16,26].

Discussion

Most importantly, our study shows how careful one needs to be in choosing *in vitro* model systems, particularly long-term cell lines, in order to obtain a suitable system for

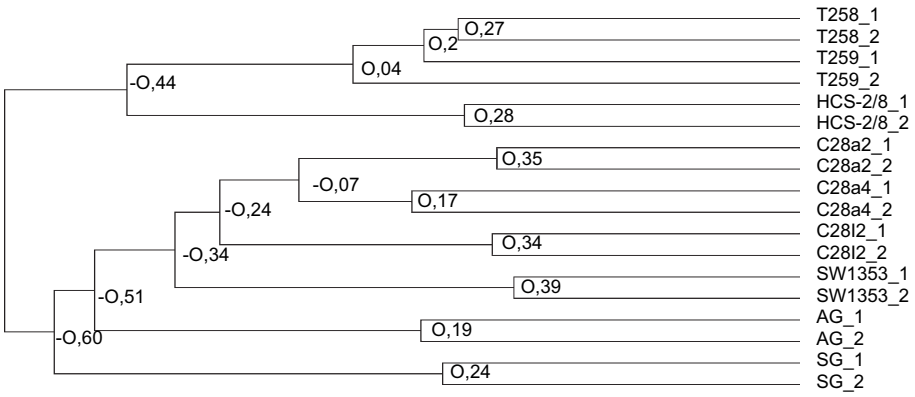

Fig. 4. Cluster analysis of different human chondrocyte cell lines (HCS-2/8, C28a2, C28A4, C28I2, SW1353, AG, SG) and primary human chondrocytes (two donors). All samples were cultured as high-density monolayer cultures and duplicate profiles were generated. An agglomerative clustering algorithm with Euclidean distance as a metric was chosen. In order to combine groups complete linkage was selected. The correlation values calculated by the Resolver software are given for each paired group.

investigating effects of interest. Our study highlights central issues related to the use of chondrocyte cell lines in cartilage research as a substitute for primary articular chondrocytes. The major issue of cell lines appears to be that they mainly proliferate: thus, they show mainly the expression of house-keeping genes involved in the basic cell machinery (e.g., ribosomal genes) as well as rather increased levels of cell cycle-associated proteins and show less expression of genes of matrix synthesis and turnover[18]. In fact, proliferative activity appears to be negatively correlated to the matrix-producing chondrocytic phenotype at least in the adult. Our study also clearly shows that reduced proliferation of HCS-2/8 is linked to the expression of the chondrocyte phenotype; thus, aggrecan or SOX9 are induced in this condition. This is similar to physiological chondrocytes, which show in most instances a decreased expression of cartilage matrix components in areas of chondrocyte proliferation in osteoarthritic cartilage degeneration[27] and reflected in many neoplasms including chondrosarcomas, which cease largely cartilage matrix formation as soon as they increase proliferative activity[28]. Also, chondrocytes cultured under proliferative conditions loose their phenotype and, thus, cartilage-specific matrix synthesis[3]. Altogether, the very low expression of most matrix degrading proteases is consistent with the very low expression of extracellular matrix genes and most likely reflects, as discussed above, the very much reduced priority in proliferating cells for synthesizing, depositing and degrading extracellular matrix.

Clearly, HCS-2/8 cells are a suitable *in vitro* model for studying aggrecan gene expression: they express it at levels similar to many adult chondrocytes and do react, e.g., to anabolic factors such as BMP-7 (own unpublished results), but they are hardly able to reflect collagen type II expression. The same is true if one is interested in investigating the regulation and activity of classical cartilage matrix degrading enzymes. Major subtypes of proteases expressed by physiological chondrocytes *in vivo* and *in vitro*[17] such as MMP-3 and the collagenases MMP-1 and MMP-13 are hardly expressed by the cells. On the other hand, in articular chondrocytes after isolation *in vitro* change their gene expression profile *in vitro* significantly. They very much increase expression of MMP-1 and MMP-13[17]. Thus, in this respect, HCS-2/8 cells might to some extent even better reflect the *in vivo* situation as isolated chondrocytes.

Altogether HCS-2/8 cells only in part reflect the *in vivo* (and *in vitro*) situation of articular chondrocytes *in situ*, though this cell line appears to be closer in many respects to physiological chondrocytes than the other chondrocytic cell lines tested in this investigation. Clustering analysis identified that the "best" (i.e., the most physiological) molecular phenotype is achieved if HCS-2/8 cells are kept in confluent culture conditions.

Cell lines are of somewhat limited use as substitutes for primary chondrocytes, but also primary chondrocytes *in vitro* are not a good substitute for chondrocytes *in vivo*[17]. They also show severe alterations in gene expression, in particular also in the expression levels of anabolic and catabolic genes ([17]; unpublished own observation). An advantage of HCS-2/8 cells compared to physiological chondrocytes is the rather high phenotypic stability of the cells compared to primary chondrocytes, which tend to loose quickly their specific expression properties after isolation from articular cartilage. In particular after long-term maintenance under culture conditions allowing the propagation of the primary cells major shifts of the expression pattern have been described[3,4]. Also the fact that using cell lines allows to avoid the donor variability one always takes into account in any experiment with primary cells. Also, primary articular chondrocytes are hardly available to the extent needed. Thus, in contrast to primary articular chondrocytes, which hardly proliferate if maintained under phenotype stabilizing conditions, HCS-2/8 cells have the advantage that they can be propagated theoretically to an unlimited extent. Overall, a successful approach will have to select one of the available chondrocyte cell lines and to validate findings obtained using primary chondrocytes or intact human articular cartilage. This allows to establish a reproducible *in vitro* model, which enables the use of large numbers of cells, and subsequently to relate back to the physiologic situation. This will enable a realistic use of the versatile tools and allow a more reliable interpretation of the results obtained in such studies.

Our data are in line with previous investigations showing, e.g., a strong expression of CTGF[29] as well as aggrecan[5,8] in HCS-2/8 cells comparable to primary human articular chondrocytes, but we could not confirm other data such as the expression of collagen type II mRNA by these cells as reported previously[5]. This might be related to culture

conditions (e.g., other serum batches) or the fact that the present experiments were performed using passage 50–55 cells while previous studies were performed with passage 20–40 cells ([5]; Takigawa, unpublished observations). Therefore, whether this discrepancy is due to a shift of the cellular phenotype during many passages or slightly different culture conditions is unclear at the moment. Clearly, this issue requires further investigations, but, as confirmed in this study, chondrocytic cells are very much dependent on the exact culture conditions.

Another aspect not explicitly dealt with in this paper is obviously the use of isolated chondrosarcoma derived cell lines for investigating the nature and properties of cartilaginous neoplasms and their cells. However, no extensive data on the expression pattern in these neoplasms are so far available. Clearly, neoplastic chondrocytes except in very highly differentiated lesions do also proliferate[5] much more strongly than adult articular chondrocytes. Interestingly, neoplastic chondrocytes appear to express more likely aggrecan and loose more often the capability of expressing collagen type II[5,30], a feature presumably reflected by the expression pattern found in HCS-2/8 cells. Clearly, the in-depth analysis of expression data of HCS-2/8 cells by cancer geneticians will open up new insights into the biology of neoplastic chondrocytes, but this was not in the range of this investigation.

This study paints a molecular portrait of a frequently used chondrocyte cell line which will help to direct further studies in the field, not only by the data explicitly discussed in this paper, but even more importantly through the full dataset available online allowing the individual researcher to exploit the dataset with a specific question in mind. Thus, our paper together with others will allow a more specific use of this and other chondrocytic cell lines for replacing physiological chondrocytes in a manner that does not implicate an intolerable loss of the physiological context, but allows expanding our knowledge about cartilage cell physiology and pathophysiology in optimized and feasible *in vitro* systems.

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References

1. Aigner T, Hemmel M, Neureiter D, Gebhard PM, Zeiler G, Kirchner T, *et al*. Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritic human articular knee cartilage: a study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage. Arthritis Rheum 2001;44: 1304–12.
2. Benya PD, Padilla SR, Nimni ME. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell 1978; 15:1313–21.
3. von der Mark K, Gauss V, von der Mark H, Müller PK. Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature 1977;267:531–2.
4. Stokes DG, Liu G, Coimbra IB, Velazquez S, Crowl RM, Jimenez SA. Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum 2002;46:404–19.
5. Takigawa M, Tajima K, Pan H-O, Enomoto M, Kinoshita A, Suzuki F, *et al*. Establishment of a clonal human chondrosarcoma cell line with cartilage phenotypes. Cancer Res 1989;49:3996–4002.
6. Simbulan-Rosenthal CM, Rosenthal DS, Iyer S, Boulares AH, Smulson ME. Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly (ADP-ribose) polymerase in the early stages of apoptosis. J Biol Chem 1998;273:13703–12.
7. Geiler G, Riedel U. Detection by the PAP technique of lysozyme-containing synoviocytes and their quantity in rheumatoid and osteoarthritis. Histochem J 1985;17: 562–3.
8. Tsuji M, Funahashi S, Takigawa M, Seiki M, Fujii K, Yoshida T. Expression of c-fos gene inhibits proteoglycan synthesis in transfected chondrocyte. FEBS Lett 1996;381:222–6.
9. Nakanishi T, Nishida T, Shimo T, Kobayashi K, Kubo T, Tamatani T, *et al*. Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. Endocrinology 2000;141:264–73.
10. Nishida T, Nakanishi T, Shimo T, Asano M, Hattori T, Tamatani T, *et al*. Demonstration of receptors specific for connective tissue growth factor on a human chondrocytic cell line (HCS-2/8). Biochem Biophys Res Commun 1998;247:905–9.
11. Eguchi T, Kubota S, Kondo S, Kuboki T, Yatani H, Takigawa M. A novel cis-element that enhances connective tissue growth factor gene expression in chondrocytic cells. Biochem Biophys Res Commun 2002;295:445–51.
12. Yosimichi G, Kubota S, Hattori T, Nishida T, Nawachi K, Nakanishi T, *et al*. CTGF/Hcs24 interacts with the cytoskeletal protein actin in chondrocytes. Biochem Biophys Res Commun 2002;299:755–61.
13. Sakai T, Kambe F, Mitsuyama H, Ishiguro N, Kurokouchi K, Takigawa M, *et al*. Tumor necrosis factor alpha induces expression of genes for matrix degradation in human chondrocyte-like HCS-2/8 cells through activation of NF-kappaB: abrogation of the tumor necrosis factor alpha effect by proteasome inhibitors. J Bone Miner Res 2001;16:1272–80.
14. Hattori T, Fujisawa T, Sasaki K, Yutani Y, Nakanishi T, Takahashi K, *et al*. Isolation and characterization of a rheumatoid arthritis-specific antigen (RA-A47) from a human chondrocytic cell line (HCS-2/8). Biochem Biophys Res Commun 1998;245:679–83.
15. Block JA, Inerot SE, Gitelis S, Kimura JH. Synthesis of chondrocytic keratan sulphate-containing proteoglycans by human chondrosarcoma cells in long-term cell cultures. J Bone Joint Surg 1991;73A:647–658.
16. Goldring MB, Birkhead JR, Suen L-F, Yamin R, Mizuno S, Glowacki J, *et al*. Interleukin-1-modulated gene expression in immortalized human chondrocytes. J Clin Invest 1994;94:2307–16.
17. Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E, Aigner T. Relative messenger RNA expression profiling of collagenases and aggrecanases in human

articular chondrocytes *in vivo* and *in vitro*. Arthritis Rheum 2002;46:2648–57.
18. Finger F, Schörle CM, Zien A, Gebhard PM, Goldring MB, Aigner T. Molecular phenotyping of human chondrocyte cell lines T/C-28a2, T/C-28a4, C-28/I2. Arthritis Rheum 2003;48:3395–403.
19. Gebhard PM, Gehrsitz A, Bau B, Söder S, Eger W, Aigner T. Quantification of expression levels of cellular differentiation markers does not support a general shift in the cellular phenotype of osteoarthritic chondrocytes. J Orthop Res 2003;21:96–101.
20. Rajagopalan D. A comparison of statistical methods for analysis of high density oligonucleotide array data. Bioinformatics 2003;19:1469–76.
21. Zhu J, Pan H-O, Suzuki F, Takigawa M. Proto-oncogene expression in human chondrosarcoma cell line: HCS-2/8. Jpn J Cancer Res 1994;85:364–71.
22. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. Sox9 is required for cartilage formation. Nat Genet 1999;22:89.
23. Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Pöschl E. Sox9 expression does not correlate with type II collagen expression in adult articular chondrocytes. Matrix Biol 2003;22:363–72.
24. McCright B, Virshup DM. Identification of a new family of protein phosphatase 2A regulatory subunits. J Biol Chem 1995;270:26123–8.
25. Bachner D, Ahrens M, Schroder D, Hoffmann A, Lauber J, Betat N, *et al*. Bmp-2 downstream targets in mesenchymal development identified by subtractive cloning from recombinant mesenchymal progenitors (C3H10T). Dev Dyn 1998;213:398–411.
26. Goldring MB, Birkhead JR, Sandell LJ, Kimura T, Krane SM. Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. J Clin Invest 1988;82:2026–37.
27. Aigner T, Vornehm SI, Zeiler G, Dudhia J, von der Mark K, Bayliss MT. Suppression of cartilage matrix gene expression in upper zone chondrocytes of osteoarthritic cartilage. Arthritis Rheum 1997;40:562–9.
28. Aigner T, Muller S, Neureiter D, Illstrup DM, Kirchner T, Bjornsson J. Prognostic relevance of cell biologic and biochemical features in conventional chondrosarcomas. Cancer 2002;94:2273–81.
29. Nakanishi T, Kimura Y, Tamura T, Ichikawa H, Takigawa M, Sugimoto T. Cloning of a mRNA preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF) mRNA. Biochem Biophys Res Commun 1997;234:206–10.
30. Aigner T. Towards a new understanding and classification of chondrogenic neoplasias of the skeleton—biochemistry and cell biology of chondrosarcoma and its variants. Virchows Arch 2002;441:219–30.